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This research investigates the role of the serine integral membrane protease seprase in human breast cancer cell invasion. The project goals are to identify and characterize a full-length cDNA for human breast cancer seprase and to investigate the contribution of seprase to the metastatic potential of breast cells. Progress made in the first year of this four year project is reported. Chicken embryo cDNA libraries have been prepared and screened with the polyclonal antibody against chicken embryo seprase that intensely labels human breast cancer cells. This probe identified a novel gene encoding a protein of unknown function. A reverse transcriptase-polymerase chain reaction (RT-PCR) strategy for cloning the seprase gene was developed based on conserved DNA sequences of human nonclassical serine proteases. A 250 bp cDNA with 85 % homology to catalytic domain of the family of serine integral membrane proteases that includes seprase was produced by RT-PCR of chicken embryo RNA. This cDNA identified thirteen clones when used to screen cDNA libraries and the clones are being characterized. Monoclonal antibodies were produced that will be useful for screening cDNA expression libraries and for purifying seprase to obtain amino acid sequences. The cell-mediated proteolysis and invasion assays needed for future work on this project were established.

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Thomas J. Lally, Jr. 9/29/97

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Table of Contents

Introduction

This report describes progress made in the first year of a four year project entitled "Role of seprase in breast cancer invasion". Seprase is a serine integral membrane protease (1) first identified in human melanoma cells (2) and chicken embryo fibroblasts transformed by Rous sarcoma virus (3). This year, a cDNA encoding human melanoma seprase was cloned and sequenced (1). The sequence reveals that seprase is a member of a family serine integral membrane proteases that includes fibroblast activation protein-α[1] (FAP-α) (4), and dipeptidyl peptidase IV (DPPIV) (5, 6). Seprase is known to degrade gelatin (1-3) and is thought to facilitate erosion of the extracellular matrix thereby promoting invasion of malignant cells. Supporting this concept is the association of seprase overexpression with the invasive phenotype and its concentration on the invadopodial membranes of human melanoma cells (2, 7). Moreover, accumulation of seprase on the surface of invadopodial membranes is stimulated by ligation of $\alpha_6\beta_1$ integrin on the melanoma cell surface (8). The localization of seprase to invadopodia supports a role for seprase in degrading extracellular matrix and facilitating tumor cell invasion because invadopodia are specialized protrusions of the plasma membranes of invasive cells that contact and degrade extracellular matrix (9, 10). Invadopodia can cause proteolysis of intact fibroblast extracellular matrices, type I collagen, type IV collagen, laminin, and fibronectin (3). Several malignant cell types are known to use invadopodia to degrade extracellular matrix including, human melanoma cells (2, 7, 8), Rous sarcoma virus-transformed chicken embryo fibroblasts (3, 9, 11) and human breast cancer cells (12, 13; see appendix Kelly et al, 1997). The fact that malignant human breast cells degrade extracellular matrix with invadopodia suggested that seprase might have a role in promoting the invasive spread of human breast cancer.

Seprase is overexpressed by invasive human breast cancer. This conclusion is based on three lines of evidence originally presented as preliminary data to support this research proposal. Specifically, a seprase-specific polyclonal antibody produced using affinity-purified chicken embryo seprase intensely labels malignant breast cells but not normal breast epithelia or stromal cells. In addition, seprase activity was detected by zymography in extracts of human breast cancer tumors and the seprase activity was five times greater than that of chicken embryo seprase as determined using a ^{3}H-gelatin substrate. Finally, seprase activity was detected by zymography in extracts of human breast cancer cell lines but not in extracts of a

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[1]Abbreviations used are: FAP-α, fibroblast activation protein-α; DPPIV, dipetidyl peptidase IV; RT-PCR, reverse transcriptase-polymerase chain reaction; F19, mouse monoclonal antibody to FAP-α; FPLC, fast pressure liquid chromatography

normal human breast cell line. Thus we proposed that seprase has an important role in breast cancer cell invasion. To investigate the role of seprase in breast cancer cell invasion we proposed these specific aims:

1) Identify and characterize a full-length cDNA for human breast cancer seprase.
2) Investigate the contribution of seprase to the metastatic potential of breast cells.

Progress made towards completing these objectives is described in the body of this report in relation to the statement of work submitted with the original proposal. The research is progressing on schedule. Our research suggests that human breast cancer seprase is related to but distinct from the seprase identified in human melanoma cells. The major areas of progress towards the first specific objective include our identification of putative seprase-specific cDNA clones using a reverse transcriptase-polymerase chain reaction (RT-PCR) strategy and production of new seprase-specific cDNA and monoclonal antibody probes. Progress has also been made towards investigating the contribution of seprase to the metastatic potential of breast cells, the second goal of the project. In this regard, a completed study showing that human breast cancer cells require functional invadopodia to degrade extracellular matrix and invade is included in the appendix. This study documents the initial characterization of the proteolytic and invasive behaviors of the human breast cell lines that will be transfected with seprase cDNA when it becomes available. The study also establishes the proposed cell-mediated matrix proteolysis assay and *in vitro* invasion assay as well as provides baseline determinations of these parameters in the human breast cells.

Body

Varsha Kaushal, Ph.D., Research Assistant Professor in the Department of Pathology at the University of Arkansas for Medical Sciences joined the laboratory at the start of this project in September, 1996. Dr Kaushal is an experienced molecular biologist who has cloned other genes (14) and is now pursuing the goal of identifying and characterizing the seprase gene in chicken embryo and human breast cancer libraries.

SOW Task 1. Months 1-3: Produce cDNA expression library with mRNA purified from human breast tumors.

I) Isolation of mRNA from human breast cancer tumors. RNA has been isolated and mRNA has been prepared from several samples of human breast cancers following surgical resection of the patients tumors. To date, the RNA obtained from surgical specimens of human patients has been highly degraded as judged by the poor resolution of the rRNA on agarose gels. We are still hoping to obtain high quality RNA from breast cancers removed directly from human patients and have been taking steps to better coordinate specimen collection. Specifically, we are working to get permission to collect and snap-freeze the specimens with liquid nitrogen in the operating room immediately upon removal from the patient.

II) **Isolation of mRNA from human breast cancer tumors grown in nude mice.** We have grown human breast cancer tumors in nude mice to have better control over surgical removal and snap-freezing of the tumors. High quality RNA has been isolated from tumors of the MDA-MB-231 human breast cell line (ATCC, Rockville, MD) as determined by agarose gel electrophoresis. These tumors also had seprase activity as judged by zymography of Triton X-100 detergent extracts of the tumor tissue. The MDA-MB-231 cell line is a model of a highly malignant human breast cancer and the cells lack receptors for estrogen, have vimentin intermediate filaments, are invasive in *in vitro* and *in vivo* invasion assays. We currently have tumors of MCF-7 human breast cancer cells growing in nude mice to harvest RNA. These cells serve as a model for breast cancers with cells that express estrogen receptors and do not have vimentin intermediate filaments but only those composed of keratin. Both these cells lines have been shown to have seprase activity by zymography.

III) **Chicken embryo cDNA libraries.** Screening of chicken embryo cDNA expression libraries was proposed and initiated because chicken embryos are a good source of seprase and the polyclonal antibody originally used to identify seprase in human breast cancer was producing using purified chicken embryo seprase as the immunogen. A chicken embryo cDNA expression library in λ gt11 (Clontech) was purchased and screened using the polyclonal antibody directed against seprase. All

twenty immunoreactive clones identified yielded an insert of approximately 800 bp in length. This uniformly short insert size led us to believe that the library did not contain larger cDNA inserts. Therefore we produced our own chicken embryo cDNA library in λ ZAP Express (Stratagene) and using the 800 bp cDNA as a probe, identified inserts ranging from 800 bp to 1.9 kb. Characterization of these sequences revealed that we have isolated a novel gene that does not encode a protease (described under task 3 below). To identify the seprase cDNA, we have transferred our effort towards using a RT-PCR strategy that is based on published DNA sequences of melanoma seprase (1), FAP-α (4), and DPPIV (5, 6) as discussed below.

SOW Task 2. Months 1-36: Produce additional seprase-specific probes for screening the cDNA expression library.

I) Oligonucleotide probes based on seprase, fibroblast activating protein-α (FAP-α) and dipeptidyl peptidase IV (DPPIV) sequences. This year, another group cloned and characterized a seprase cDNA from the LOX human melanoma cell line (1). They produced a 2.4 kb seprase cDNA from LOX mRNA using FAP-α oligonucleotide primers and RT-PCR. This strategy was taken because sequence analysis of three internal peptides of the 97 kDa seprase subunit had up to 87.5 % homology to FAP-α (1). This work has shown that the proteolytically active 170 kDa form of human melanoma seprase is a dimer of the 97 kDa subunit (1).

The seprase that is overexpressed by human breast cancer cells and its chicken embryo homolog may represent a novel member of the seprase, FAP-α, DPPIV family of integral membrane proteases. It is not precisely related to seprase or FAP-α because the FAP-α oligonucleotide primers used by others to RT-PCR amplify a seprase cDNA from human melanoma cell RNA (1) did not produce an amplification product when used for RT-PCR to amplify a cDNA from RNA of the MCF-7 or MDA-MB-231 human breast cancer cells. Moreover, the FAP-α oligonucleotide primers failed to amplify a cDNA from chicken embryo RNA. A monoclonal antibody termed F19 has been produced against FAP-α (4) and the hybridoma cells producing this antibody are available through the ATCC. In contrast to our polyclonal antibody against seprase, the F19 antibody to FAP-α labels the reactive stroma of human breast cancers and not the malignant cells themselves (4). We have not detected FAP-α in MCF-7 or MDA-MB-231 cells by immunoblot analysis with F19. We conclude that the seprase activity we detect in human breast cancer extracts and in chicken embryos is composed of a related but distinct subunit in the non-classical serine protease family that includes seprase, FAP-α, and DPPIV.

An alternative RT-PCR based cloning strategies was investigated by comparing the sequences of other seprase-family proteases. The catalytic domains of FAP-α,

DPPIV, DPPX and seprase consists of a serine-aspartate-histidine triad spanning approximately 100 residues with regions of conserved sequences (1, 4-6). This information was used to design degenerate oligonucleotide primers for conserved sequences in the catalytic site of these enzymes. A 250 bp cDNA fragment was amplified by RT-PCR of chicken embryo mRNA. The 250 bp cDNA was subcloned and sequenced. Sequence analysis revealed 85 % homology to known serine integral membrane proteases. The 250 bp cDNA was used to probe the cDNA library in the λ ZAP Express vector. Thirteen positive clones have been identified, isolated, and plaque purified. We are in the process of sequencing and characterizing these clones.

II) Production of monoclonal antibodies directed against seprase.

Chicken embryo seprase purification: Peterson ♂-Hubbard ♀ chicken eggs were obtained from Keith Smith Farms (Hot Springs, AR) and grown for nine days at 39° C with slow rotation in a humidified incubator (G.Q.F. Manufacturing Co., Savannah, GA). Embryos were removed, rinsed 3 times with cold PBS and used immediately or stored at -80° C.

For purification of seprase from 240 (429 g) or more embryos, various peripheral membrane proteins were removed by sequential extractions. First the embryos were homogenized in 10 volumes of 10 mM Tris, 5 mM EDTA, pH 7.6. The insoluble material was collected by centrifugation and extracted with the same, low ionic strength buffer for 30 min at 37° C. The insoluble material was then extracted with 10 mM Tris-HCl, 0.6 M NaCl, 5 mM EDTA, pH 7.6 for 1 h at 4° C. After each extraction the insoluble material was collected by centrifugation and the supernatant was discarded. The pellet was extracted with 2.6 % Triton X-114, 10 mM Tris-HCl, 5 mM EDTA, pH 7.6 the supernatant liquid remaining after centrifugation was used to continue purifying the protease.

The detergent extract was loaded (70 ml/h) onto an 80 ml DEAE-cellulose column (Whatman DE52, Whatman International) equilibrated in 0.5 % Triton X-100, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5. The column was washed with 560 ml of equilibration buffer, then eluted at 20 ml/h with 560 ml of a continuous gradient ranging from 0 to 400 mM NaCl. Fractions (7 ml) were collected and those with seprase activity were identified by zymography and pooled for purification by WGA agarose (10 ml). The WGA column was washed with 50 ml of 0.5 % Triton X-100, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5. The activity was eluted from the WGA column with 10 % (w/v) GlcNAc in this same buffer and a 6 ml fraction followed by three consecutive 10 ml fractions were collected. The last three fractions were pooled and purified by Fast Pressure Liquid Chromatography (FPLC; Pharmacia-LKB, Uppsala, Sweden) using the mono-Q anion exchange and Superdex 200 gel filtration columns. The seprase activity eluted from the mono-Q column

over several fractions. Fractions in group 1 had lower activity of highly purified seprase. Fractions in group 2 had the higher seprase activity and a proteolytically inactive 120 kDa protein not found in the first two fractions. These groups were concentrated and separately subjected to gel filtration using the FPLC and Superdex 200. Seprase activity was detected in the first 3 ml of included volume eluting off the Superdex 200 column for both groups 1 and 2. The 3 ml was concentrated 10 fold using centricon 30 filter concentrators and analyzed by SDS-PAGE and zymography. Samples of purified seprase from groups 1 and 2 were used to immunize mice.

Generation of monoclonal antibodies to chicken embryo seprase. Female Balb c mice were injected IP and SC with purified seprase groups 1 or 2 emulsified 1:1 in Freund's complete adjuvant. At 6 weeks after the initial injection, a boost was administered with the same antigens emulsified 1:1 in Freund's incomplete adjuvant. The mice were bled one and two weeks after the boost and the serum tested for ability to precipitate seprase activity from chicken embryo extracts. Serum from a mouse injected with seprase group 2 was able to precipitate seprase activity from detergent extracts of chicken embryo tissues but serum from a mouse injected with seprase group 1 was unable to immunoprecipitate seprase activity. Immunoblot analysis of partially purified chicken seprase revealed immunoreactivity with a protein at 160 kDa corresponding to the proteolytically active form of seprase. Thus, this mouse seemed to have a high titer serum directed against active chicken embryo. A final boost of seprase group 2 was administered to the mouse and then it was sacrificed to obtain its spleen for producing hybridomas.

Non-secreting mouse myeloma cells (P3/NSI/1-Ag4, ATCC, Rockville, MD) were fused to splenocytes from the mouse immunized with chicken embryo seprase group 2 using polyethylene glycol. The hybridomas were suspended in HAT selection medium, plated into 96 well culture plates, and allowed to grow. Between 10 and 14 days after fusion the clones that grew (586) were transferred from the 96 well plates to 24 well plates so that they could be expanded to provide enough antibody in the conditioned growth medium for successful immunoprecipitation of seprase. Hybridoma supernatants were screened by immunoprecipitation for IgG that react with seprase activity as judged by gelatin zymography of the immune precipitates. Two clones (56 and 8D2) were discovered that precipitated seprase activity in more than one immunoprecipitation assay. These were subcloned by limiting dilution, and the subclones tested for ability to precipitate seprase. Subclones 56.E6 and 8D2.E2 were notably successful in precipitating seprase activity. The detailed characterization of the antigens recognized by these two antibodies is currently ongoing. These monoclonal antibodies will be useful for further screening of chicken embryo and human breast cancer cDNA libraries to identify a full length seprase cDNA.

SOW Task 3. Months 3-18: Identify clones with full-length seprase cDNA inserts. The 800 bp insert identified in the λ gt11 chicken embryo cDNA library using our polyclonal antibody against seprase was used as a probe to screen our λ ZAP Express chicken embryo cDNA library and for Northern blot analysis of RNA isolated from chicken embryos. The 800 bp probe identified clones in the λ ZAP Express chicken embryo cDNA library with inserts ranging in size from 0.8 kb to 1.9 kb. Moreover, Northern analysis of chicken embryo mRNA revealed hybridization of the probe to mRNAs of 1.8 and 3.5-4 kb. Sequence analysis of the 800 bp probe revealed partial homology to GTP-GDP exchange motif of elongation factors in an approximately 450 bp region at the 3' end but the remaining 350 bp at the 5' end showed no sequence homology. Subsequent sequence analysis of the entire 1.8 kb cDNA isolated by immunoscreening with the polyclonal antibody directed against seprase did not show any significant homologies to any known sequences and hence represents a novel gene. The function of the product of this gene is unknown; however, it is possible that it is a protein associated with the seprase complex that may be involved in regulating seprase activity.

SOW Tasks 4 & 5 Confirm full-length seprase cDNA clones and produce stably transfected malignant and normal breast cells that overexpress assembled, active seprase to the cell surface. Task 4 months 4-24; Task 5 months 18-36

We have not yet isolated a putative full-length seprase clone. Task four can not be completed until a putative full-length cDNA is identified. Similarly, task 5 requires isolated, characterized and confirmed full-length seprase cDNA. No progress has been made on this task yet. However, we are still within the anticipated time frames for both of these tasks.

Preparations for future completion of SOW Tasks 6 & 7.
Task 6. Months 24-48: Determine effects of seprase overexpression on cell-mediated matrix proteolysis.
Task 7. Months 30-48: Determine effects of seprase overexpression on breast cell invasion of extracellular matrix.

Evaluation of human breast cancer cells for invadopodial proteolysis of extracellular matrix and invasiveness. We have completed a study investigating the role of invadopodial proteolysis of extracellular matrix in invasion by human breast cancer cells (See Appendix, submitted manuscript "Invadopodial proteolysis of extracellular matrix facilitates human breast cancer cell invasion and is mediated by matrix metalloproteinases"). The study finds that invadopodial proteolysis of extracellular matrix is positively correlated with invasive behavior. Moreover, inhibiting invadopdial proteolysis of extracellular matrix with the matrix metalloproteinase inhibitor batimastat, decreases the invasiveness of malignant breast cells. These findings indicate that invadopodial proteolysis of extracellular matrix greatly

facilitates invasion by human breast cancer cells.

Conclusions:

Our research suggests that human breast cancer seprase is related to but distinct from the seprase identified in human melanoma cells. The research completed to date has identified putative seprase-specific cDNA clones using a RT-PCR strategy and produced new seprase-specific cDNA and monoclonal antibody probes. In addition, completed research shows that human breast cancer cells require functional invadopodia to degrade extracellular matrix and invade (Appendix). Baseline determinations of the extracellular matrix-degrading and invasive behaviors were made for HBL-100 normal human breast cells as well as MCF-7 and MDA-MB-231 malignant human breast cells. The study documents the initial characterization of the proteolytic and invasive behaviors of the human breast cell lines that will be transfected with the seprase cDNA. In executing this study, the cell-mediated matrix proteolysis assay and *in vitro* invasion assay needed for the completion of this research project were established.

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Appendix: Submitted manuscript (Note: References cited in the manuscript refer to a separate reference list that is included within the manuscript.)

Invadopodial proteolysis of extracellular matrix facilitates human breast cancer cell invasion and is mediated by matrix metalloproteinases[2,3].

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Invadopodial MMPs facilitate invasive behavior

Keywords: Invadopodia, (BB-94) batimastat, invasion, MMP, metastasis, fibronectin

Abstract

Breast cancer cell lines vary in invasive behavior and one cell line (MDA-MB-231) proteolytically degrades extracellular matrix with invadopodia (Chen et al, 1994, *Breast Can. Res. and Treat.* **31**:217-226) and is very invasive (Thompson et al, 1992, *J. Cell. Physiol.* **150**:534-544). Invadopodial proteolysis of extracellular matrix is thought to be necessary for invasion; however, this has not been directly demonstrated. This study evaluates normal (HBL-100) and malignant (MCF-7, MDA-MB-231) breast cells for invadopodial proteolysis of extracellular matrix and invasive behavior. We report that invadopodial proteolysis of immobilized fibronectin is positively correlated with invasion of cells into type I collagen gels. Moreover, reducing the proteolytic activity of invadopodia with the metalloproteinase inhibitor, batimastat (BB-94), also decreases invasion indicating that breast cancer cell invasion is dependent upon proteolytically active invadopodia.

Invadopodial MMPs facilitate invasive behavior

Introduction

Invasive tumor cells have increased levels of a variety of extracellular matrix-degrading proteases that allow them to traverse complex basement membrane and stromal matrices (reviewed in: (1-3). Generally, the matrix-degrading proteases are secreted by tumor and stromal cells as inactive zymogens that require association with the tumor cell surface to become activated and capable of degrading extracellular matrix (1, 2). The cellular sites of abnormally high extracellular matrix-degrading activity are plasma membrane extensions termed "invadopodia" because of their role in breaking down the extracellular matrix and their association with invading cells (4). This is known because invasive cells growing on monolayers of fluorescently labeled extracellular matrix proteins produce fluorescence-negative regions underneath the cells where matrix proteolysis has occurred (5, 6). The proteolysis is discretely focused and corresponds to areas where invadopodia extend from cell surfaces and contact the matrix (4-6). Invadopodia can degrade multiple extracellular matrix proteins including intact matrices produced by fibroblasts, fibronectin, laminin, type IV collagen, and type I collagen (6). A variety of invasive cells exhibit invadopodia including human melanoma cells (7), transformed chicken fibroblasts (4-6), and human breast cancer cells (8, 9). Invadopodial proteolysis of extracellular matrix is thought to facilitate tumor cell invasion into extracellular matrix (3, 4, 10).

Invadopodial MMPs facilitate invasive behavior

Matrix metalloproteinases (MMPs[4]) are important mediators of invadopodial degradation of extracellular matrix because an inhibitor of MMPs (NP-20) decreases invadopodial degradation of type I collagen by Rous sarcoma virally transformed chicken embryo fibroblasts (11). MMPs are a family of structurally related enzymes that together can degrade all components of the extracellular matrix and are known to be important in tumor cell invasion (1, 2). MMPs are synthesized as inactive proenzymes that require cleavage of the pro-peptide for activation of their proteolytic activities. Proteolytically active MMP-2 (gelatinase A) and MT1-MMP (membrane type 1-matrix metalloproteinase or MMP-14) are located on invadopodial membranes suggesting that these enzymes are important mediators of invadopodial extracellular matrix degradation (11, 12). MT1-MMP is activated by furin-like enzymes prior to its expression on the cell surface (13, 14) where it serves as a potent activator of latent MMP-2 (15, 16). Given the wide range of extracellular matrix substrates degraded by invadopodia, other proteases including other MMPs are likely to have a role in proteolysis of extracellular matrix by invadopodia. Seprase, is an invadopodial serine protease that is integral to the plasma membrane, requires non-covalent oligomerization of subunits for activity and can degrade gelatin (7, 17, 18). Seprase is not restricted to invadopodial membranes and is

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[4]Abbreviations used are: MMP, matrix metalloproteinase; MT1-MMP, membrane type-1 matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; PBS, phosphate buffered saline; BSA, bovine serum albumin, DMSO, dimethyl sulfoxide

widely distributed on the plasma membrane; however, in human melanoma cells it is markedly concentrated to invadopodia after ligation of $\alpha_6\beta_1$ integrins by laminin G peptides suggesting a possible role for integrin-mediated signalling in coordinating invadopodial proteolysis of the extracellular matrix (19). The precise function of seprase in invadopodial degradation of extracellular matrix proteins is unknown, but it may function to modulate the activity of other "work horse" proteases such as plasminogen and MMP-2 (3).

This study was performed to determine if invadopodial proteolysis of extracellular matrix plays an important role in facilitating human breast cell invasion. Previously, it was shown that the invasive MDA-MB-231 human breast cancer cells use invadopodia to degrade films of fluorescent extracellular matrix molecules covalently attached to glutaraldehyde crosslinked gelatin (8, 9). Others have shown that human breast cancer cell lines vary in their ability to invade into matrigel and MDA-MB-231 were identified as the most invasive human breast cancer cell line tested *in vitro* (20). It also was shown that human breast cancer cell lines varied in local invasion through the peritoneum when injected into the mammary fat pads of nude mice and MDA-MB-231 cells were again identified as most invasive because they formed tumors that had the highest frequency of malignant cells invading through the peritoneum (20). In contrast, MCF-7 human breast cancer cells were moderately invasive through matrigel in Boyden chamber assays and although these cells formed tumors when injected into the mammary fat

pads of nude mice, the tumor cells did not invade through the peritoneum (20). Here we investigated human breast cell lines for invadopodial function as determined by degradation of fluorescently labeled or radiolabeled fibronectin and for invasiveness using type I collagen gels. The MMP inhibitor batimastat was used to inhibit the function of invadopodia to thereby investigate the role of invadopodia in breast cancer cell invasion.

Invadopodial MMPs facilitate invasive behavior

Materials and Methods

Cell culture: Human breast adenocarcinoma cell lines MDA-MB-231, MCF-7, and the normal breast cell line HBL-100 were obtained from American Type Culture Collection (Rockville, MD). All cell lines were maintained in Eagle's minimal essential medium (Gibco-BRL, Gaithersburg, MD), supplemented with 10 % heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA), 10 μg/ml bovine insulin (Sigma, St. Louis, MO), 10 μg/ml glutamine (Gibco-BRL, Gaithersburg, MD), 1 % penicillin-streptomycin (Gibco-BRL, Gaithersburg, MD), 2 X 10^{-8} mM B-estradiol (Sigma, St. Louis, MO) and maintained at 37°C and 95 % air.

Fluorescent fibronectin substrates for determining invadopodial proteolysis of extracellular matrix: Two assays were used to determine invadopodial proteolysis of extracellular matrix. The first has been previously described (5, 6) and relies on growing the cells on fluorescein isothiocyanate-labeled fibronectin covalently bound to a glutaraldehyde-crosslinked gelatin film attached to a glass coverslip and observing fluorescence-negative regions underneath cells that have degraded the matrix. Human plasma fibronectin (Becton Dickinson Labware, Medford, MA) was coupled to fluorescein isothiocynate (Research Organics, Cleveland, OH) according to the manufacturers instructions. The assays reported here were allowed to proceed for 24 h or 72 h at 37° in 95% air prior to fixation and preparation for fluorescence microscopy.

Fluorescence microscopy: The cells were fixed, stained with rhodamine

phalloidin (Molecular Probes, Inc., Eugene, OR) as described earlier (6). The cells were observed using 40 X, 60 X and 100 X objectives of a Nikon LABOPHOT microscope equipped for epifluorescence photomicroscopy and images were recorded using Ilford HP5 400 ASA black and white film. To quantify invadopodial matrix proteolysis, ten microscopic fields on each coverslip were randomly selected and the fluorescence of the fibronectin film visualized with the 40 X objective and scored for the presence or absence of matrix proteolysis as judged by the presence of focal fluorescence negative spots in areas where the matrix was degraded (5, 6, 8). The results are presented as the average number of fields positive for matrix degradation per ten fields examined.

^{125}I-fibronectin substrates for determining invadopodial proteolysis of extracellular matrix: The second invadopodial proteolysis assay utilized ^{125}I fibronectin as the substrate and required the following procedures: removal of proteases from the fetal bovine serum used in the growth medium, iodination of the fibronectin, coupling the ^{125}I-fibronectin to crosslinked gelatin films, determination of ^{125}I-fibronectin bound to the substrate, extensive washing to remove free ^{125}I-fibronectin, and determination of invadopodial proteolysis.

Removal of interfering extracellular matrix-degrading proteases from fetal bovine serum: Gelatin Sepharose chromatography was used to remove MMPs and TIMPs from fetal bovine serum (21, 22). A 10 ml gelatin-Sepharose (Pharmacia Biotech, Uppsala, Sweden) was equilibrated with the binding buffer 20 mM Tris,

0.5 M NaCl, 1.0 mM $CaCl_2$, 10% glycerol(v/v), 0.05% BRIJ-35 (v/v), 0.02 % NaN_3 (v/v), pH 7.6. MMPs and TIMPs in fetal bovine serum (heat inactivated) were bound to the column by loading the serum (50 ml) onto the column at 35 ml/h. The void volume was discarded, and the flow-through, depleted of MMPs and TIMPs but containing the other serum components, was collected and forced through a sterile filter with 0.2 μm pores. Gelatin zymography of breast cancer cell growth medium made 10 % (v/v) with respect to fetal bovine serum revealed no MMP activity (not shown). Fetal bovine serum depleted of MMPs and TIMPs was used for the experiments reported in figures 1, and 3A.

For the batimastat (batimastat or BB-94, kindly provided by British Biotech, Inc., Oxford, UK) inhibitor studies reported in figures 2, 3B, and 5, fetal bovine serum was not only depleted of MMPs and TIMPs as described above but was also subjected to lysine Sepharose chromatography to remove the broad spectrum protease, plasmin/plasminogen that is abundant in serum (23). A 10 ml lysine Sepharose column (Pharmacia Biotech, Uppsala, Sweden) was packed and equilibrated with the same binding buffer described above. The MMP/TIMP-depleted fetal bovine serum (50 ml) was loaded onto the column at 35 ml/h. The MMP/TIMP and plasminogen/plasmin-depleted serum in the flow-through was collected and forced through a sterile filter with 0.2 μm pores. Immunoblot analysis of breast cancer cell growth medium made 10 % (v/v) with respect to MMP/TIMP and plasminogen/plasmin-depleted fetal bovine serum revealed no plasminogen or

plasmin immuno-reactivity with a goat IgG directed against bovine plasminogen (American Diagnostics, Greenwich, CT) (not shown).

Iodination of fibronectin, coupling to crosslinked gelatin and determination of cpm ^{125}I-fibronectin bound to the substrate: Fibronectin (50 μg) was iodinated with 0.5 mCi ^{125}I using chloramine T (2 mg/ml) as described (24). ^{125}I-fibronectin was separated from free ^{125}I by gel filtration using an excellulose G5 column (Pierce, Rockford, IL) that had been equilibrated in 1 mg/ml BSA and PBS. An equivalent amount ^{125}I-fibronectin in 200 μl was coupled to the glutaraldehyde crosslinked gelatin film coating each 15 mm glass round coverslip as previously described for fluorescent fibronectin (5, 6, 17). The level of ^{125}I-fibronectin used for experiments varied from 2 x 10^6 cpm to 10^{10} cpm. The ^{125}I labeled fibronectin was allowed to bind to the coverslips for at least 12 hours. At the end of coupling, 1 μl of the coupling fluid was counted and the volume of coupling fluid was measured to determine the amount of ^{125}I-fibronectin that did not bind to each coverslip.

The ^{125}I-fibronectin-coupled crosslinked gelatin films were subjected to a series of washes designed to remove any free or weakly bound ^{125}I-fibronectin and to block exposed aldehyde groups. These used 3 ml of the following solutions: 70 % ethanol (1 x 5 min, 22°), PBS (3 x 5 min, 22°), growth medium containing 10 % MMP/TIMP and plasminogen/plasmin-depleted fetal bovine serum (1 x 3 h, 37°), PBS (2 x 5 min, 22°), serum-free growth medium (1 x 24 h, 37°). A sample (10 μl) was taken to determine the level of ^{125}I-fibronectin in each wash. The total ^{125}I-

fibronectin that did not bind each coverslip was determined by adding the radioactivity (cpm) in the unbound fraction to the sum of the radioactivity (cpm) released by all of the washes. Then the amount of ^{125}I-fibronectin bound to each coverslip was determined by subtracting the total unbound radioactivity from the radioactivity originally added to the coverslip.

Determination of invadopodial matrix proteolysis using immobilized ^{125}I-fibronectin: Human breast cells were harvested using trypsin-EDTA, diluted with growth medium containing 10 % protease-depleted fetal bovine serum and washed 3 times with sterile PBS. Cells (1 X 10^5) were placed onto each coverslip in 200 μl of medium and allowed to attach to the ^{125}I-fibronectin for 1 h at 37° and then 2.8 ml of growth medium containing 10 % MMP/TIMP and plasminogen/plasmin-depleted fetal bovine serum containing no additives, DMSO, or 10 μM batimastat in DMSO was added to achieve a final volume of 3 ml in each well of the 6 well culture plate. The plates were placed back into the incubator and 50 μl aliquots were taken from each well under sterile conditions and counted at 3, 24, 48, 72 and 96 h. At each time point, 50 μl of growth media containing 10 % protease-depleted fetal bovine serum were put back into each well to maintain the 3 ml volume of the assay. The radioactivity of the aliquots was also determined on the Packard gamma counter.

The total amount of ^{125}I released into the media for each time point was calculated and added to the sum of the radioactivity in the 50 μl aliquots from the

prior time points. This value was divided by the total cpm ^{125}I-fibronectin bound to that coverslip and multiplied by 100 to give the percentage of total bound ^{125}I released into the media.

Cell viabilities: The cytotoxicity of 72 h exposure to batimastat and DMSO was investigated by performing trypan blue exclusion assays as described by others (25). A minimum of 500 cells were counted and the number of dead cells that had taken up the blue dye was recorded. The percentage of living cells is reported.

Gelatin zymography: Gelatin zymography was performed essentially as described by Heussen and Dowdle (26) with 1 mg/ml gelatin co-polymerized into SDS-PAGE that was 10 % (w/v) with respect to acrylamide.

Conditioned growth medium: To determine the protease activities released into the medium by human breast cancer cells and to investigate the inhibitory effects of Batimastat (BB-94) on those proteases, MDA-MB 231 cells were grown to high density (7 X 10^5 cells/ml) in growth medium containing 10 % (v/v) fetal bovine serum in 75 cm^2 flasks. Complete growth medium was removed and the cells washed three times with 10 ml sterile PBS. After removing the final wash, the cells were incubated in serum-free minimal essential media for 48 h at 37° in 5 % CO_2. The conditioned medium was collected, cells and debris removed by centrifugation (5,000 x *g*, 5 min), and concentrated 10 times using centricon 30 filter concentrators (Amicon, Inc., Beverly, MA). The concentrated conditioned medium was tested for proteases in the present or absence of 10 μM batimastat by

zymography.

Invasion assay: Type I collagen gel invasion assays were performed in 24 well plates as described by Liebersbach and Sanderson (27) except that the assays was allowed to proceed for 72 h. Quantification of the percentage of invading cells was achieved by first determining the number of noninvading cells that could be removed from the top of the gel with trypsin-EDTA and then counting the invading cells that were released from within the type I collagen gel by extensive collagenase digestion as described by Liebersbach and Sanderson (27). The leading front of invasion was defined as the point where the two most distantly migrating breast cancer cells were simultaneously in focus in one field under 200 X magnification. After 72 h, each gel was searched in a Z pattern and the mean distance ± standard error. was determined using the calibrated micrometer of a Nikon inverted phase contrast microscope. Within experiments, duplicate assays were performed for each cell line and each experiment was repeated at least two times.

Results

Invadopodial extracellular matrix degrading activities of human breast cell lines. The MDA-MB-231 human breast cancer cells are proteolytically active and degrade extracellular matrix substrates by an invadopodial-dependent mechanism (8, 9). Other work has shown that human breast cancer cell lines differ in invasive behavior as determined *in vitro* using a Boyden chamber invasion assay with matrigel as the barrier to invasion and the invasiveness observed *in vitro* correlates closely with *in vivo* invasive behavior observed using the nude mouse model (20). These workers identified the MDA-MB-231 cell line as one of the most invasive. The purpose of this study was to determine the role of invadopodial proteolysis of extracellular matrix in the invasion process.

The extracellular matrix-degrading activity of the human breast cell lines was investigated by growing the cells on fluorescently labeled fibronectin that was covalently coupled to a glutaraldehyde-crosslinked gelatin film. The fetal bovine serum used for growing the cells was depleted of MMPs and TIMPs by gelatin-Sepharose chromatography (21, 22) to reduce possible proteolysis of the extracellular matrix by serum proteases. HBL-100 normal human breast cells do not degrade the matrix and the fibronectin substrate remains intact and uniformly fluorescent underneath the cells after 72 h (Fig 1, A & B). MCF-7 human breast cancer cells reveal limited degradation of the fluorescent-fibronectin film, with one microscopic field in ten having the spots of decreased fluorescence that are

indicative of matrix proteolysis after 72 h (not shown). The MDA-MB-231 breast cancer cell line reveals extensive degradation of the fluorescent fibronectin films, with five microscopic fields in ten having evidence of matrix degradation after 72 h (Fig. 1, C & D). Identical relative levels of invadopodial degradation of extracellular matrix were observed when the different cell lines were grown in medium prepared with complete serum that did not have proteases removed. MDA-MB-231 invasive breast cancer invadopodial degradation of fluorescent fibronectin films was also apparent after 24 h (Fig. 2, A & B) and was inhibited by growing the cells in the presence of 0.1 μM and 1.0 μM batimastat (BB-94), an inhibitor of MMPs (Fig. 2 C-F). Neither DMSO nor batimastat were toxic to the cells during incubation periods up to 72 h or at 0.1 μM, 1.0 μM, or 10.0 μM batimastat concentrations used in this study as judged by trypan blue exclusion assays (Table 1) and by observing typical morphologies of cells growing in the presence of batimastat (Fig. 2 A, C, & E). Thus, batimastat inhibits invadopodial function without cytotoxic effects on the cells.

To quantify the extracellular matrix proteolysis, normal HBL-100 and malignant MDA-MB-231 breast cells were grown on immobilized ^{125}I-fibronectin in medium containing MMP/TIMP-depleted fetal bovine serum and the radioactivity released from the substrate by the proteolytic action of the cells was determined. MDA-MB-231 cells release 11 fold more ^{125}I-fibronectin degradation products from the substrate than HBL-100 cells after 72 h (Fig. 3A). The effect of the batimastat

on the proteolysis of extracellular matrix by the MDA-MB-231 breast cancer cell line was investigated using cells grown on radiolabeled fibronectin in medium containing fetal bovine serum that was depleted of MMPs and TIMPs by gelatin-Sepharose chromatography (21, 22) and also depleted of plasminogen/plasmin by lysine-Sepharose chromatography (23). Under these growth conditions, batimastat (10 μM) reduced the release of ^{125}I-fibronectin from the substrate by MDA-MB-231 human breast cancer cells to below that released by control experiments without cells (Fig. 3B, -□-). The matrix-degrading proteolytic activity of the MDA-MB-231 cells grown on ^{125}I-fibronectin was high (Fig. 3B, ···□···) and only slightly diminished by the DMSO vehicle (Fig. 3B, ■). Moreover, batimastat was able to inhibit the 92 kDa and 72 kDa gelatinase activities released into serum-free medium by MDA-MB-231 cells (Fig. 3, inset). The results of the fluorescent-fibronectin and radioactive-fibronectin proteolysis assays show that invadopodial proteolysis of extracellular matrix varied between human breast cell lines with HBL-100 cells least active, MCF-7 cells weakly active and MDA-MB-231 cells exhibiting the highest level of invadopodial extracellular matrix-degrading activity. In addition, the MMP inhibitor batimastat suppressed the invadopodial extracellular matrix-degrading activity of MDA-MB-231 cells.

Invadopodial extracellular matrix degrading activity correlates with invasion potential of human breast cell lines. Invasion was evaluated by seeding the cells on top of a native type I collagen gel and allowing them to interact with the gel for 72

h while growing in medium containing 10 % complete fetal bovine serum (Fig. 4). The percentage of cells that invaded into the type I collagen gels after 72 h was determined (Fig. 4A). The HBL-100 normal human breast cell line is not invasive in this assay with only 1.2 % invading cells (Fig. 4A, H100). The MCF-7 human breast cancer cell line is somewhat invasive with 3.58 % invading cells (Fig. 4A, MCF7) and the MDA-MB-231 human breast cancer cell line is the most invasive in this assay with 10.7 % invading cells (Fig. 4A, M231). The leading front of invasion is defined as the deepest level in the gel where at least two cells are simultaneously in focus and is determined at the end of the experiment using the calibrated fine focus of an inverted microscope (Fig. 4B). The HBL-100 normal human breast cell line did not invade and the leading front of cells was 100 μm or approximately one cell diameter into the gel (Fig. 4B, H100). The human breast cancer cell lines invaded into type I collagen with the leading front of MCF-7 cells approximately six cell diameters (667 μm) into the gels and the leading front of MDA-MB-231 cells was 12 cell diameters (1,233 μm) into the gels (Fig 4B, MCF7 & M231). Both the percentage of cells invading into type I collagen gels and the depth of the leading front of invasion indicate that normal HBL-100 cells are not invasive and that the malignant breast cells vary in invasive behavior with MCF-7 cells weakly invasive and MDA-MB-231 cells highly invasive (Fig. 4C & D). These invasion results coincide exactly with the invadopodial proteolytic activities of the cells discussed above. Thus, invadopodial proteolytic activity is positively

correlated with invasive behavior.

Batimastat inhibits MDA-MB-231 human breast cancer cell invasion into type I collagen gels. The correlation between invadopodial matrix-degrading activity and invasion into type I collagen gels suggested that inhibition of invadopodial matrix proteolysis would decrease invasiveness of the malignant cells. To substantiate the observed correlation, the effect on invasiveness of inhibiting invadopodial proteolysis of matrix with batimastat was investigated. The normal HBL-100 cells growing in medium containing MMP/TIMP and plasminogen/plasmin-depleted fetal bovine serum had between 1.26 % to 6.56 % of cells invading the type I collagen gels (Fig. 5, H100). Malignant MDA-MB-231 cells growing in growth medium containing MMP/TIMP and plasminogen/plasmin-depleted fetal bovine serum or the same medium with added DMSO revealed invading cell populations of between 11.7 % to 15.0 % cells relative to the total number of cells in the experiment (Fig. 5A, M231 & M231 + DMSO). Batimastat inhibits invasion of MDA-MB-231 cells into type I collagen gels (Fig. 5A). Batimastat at concentrations of 10 μM, 1.0 μM, and 0.1 μM inhibited invasion from 2.7 fold to 10 fold relative to invasion observed by control cells growing in the presence of DMSO (Fig. 5A; M231 + 10 μM Bat, M231 + 1.0 μM Bat, and M231 + 0.1 μM Bat). Moreover, there is a dose dependency within individual experiments where greater concentrations of batimastat result in greater inhibition of invasion (Fig. 5A; experiment 1 (hatched bars) compare M231 + 1.0 μM Bat with M231 + 0.1 μM Bat; experiment 2 (clear

bars) compare M231 + 10 μM Bat with M231 + 1.0 μM). Batimastat also reduced the leading front of invasion (Fig. 5B). Normal HBL-100 cells growing in growth medium containing MMP/TIMP and plasminogen/plasmin-depleted fetal bovine serum invaded between 260 μm to 280 μm into the type I collagen gels (Fig. 5B, H100). MDA-MB-231 breast cancer cells growing in growth medium containing MMP/TIMP and plasminogen/plasmin-depleted fetal bovine serum or the same medium with added DMSO revealed leading fronts of invasion ranging from 980 μm to 1120 μm (Fig. 5B; M231 & M231 + DMSO). Batimastat at concentrations of 10 μM, 1.0 μM, and 0.1 μM reduced the distance into the gel covered by cells at the leading front of invasion causing it to be undetectable or located at 520 μm (Fig. 5B, M231 + 10 μM Bat, M231 + 1.0 μM Bat, and M231 + 0.1 μM Bat). There is a dose dependency within a given experiment where greater concentrations of batimastat result in greater reduction of the distance that cells invaded into the type I collagen gel (Fig. 5B; experiment 1 (hatched bars) compare M231 + 1.0 μM Bat with M231 + 0.1 μM Bat; experiment 2 (clear bars) no leading front detected below the top of the gel at either M231 + 10 μM Bat with M231 + 1.0 μM). Together, these results indicate that matrix metalloproteinase activity is required for human breast cancer cell invadopodial proteolysis of extracellular matrix and invasion.

Discussion

Invadopodial proteolysis of extracellular matrix greatly facilitates invasion by human breast cancer cells. Two main lines of evidence support this conclusion. First, the invasion potential of breast cancer cells is directly correlated with the extracellular matrix degrading activity of their invadopodia. For each cell line tested, the relative level of invadopodial proteolysis of extracellular matrix as determined using fluorescent or radiolabeled fibronectin substrates, positively correlated with the relative level of invasiveness as judged using type I collagen gels to determine the percentage of cells invading into the gels and the distance traveled by cells at the leading front of invasion. Second, treatment of invasive MDA-MB-231 human breast cancer cells with the MMP inhibitor batimastat negatively impacted invadopodial function as revealed by reduced cell-mediated proteolysis of immobilized fibronectin. Batimastat also reduced MDA-MB-231 cell invasion into type I collagen gels as reflected by the reduced percentages of invading cells and lower distances invaded into the type I collagen gels as compared to uninhibited cells. Batimastat inhibition of invadopodial matrix proteolysis and invasion is apparently due to its inhibition of MMP proteolytic activities because batimastat effectively inhibited two gelatinase activities at 92 kDa and 72 kDa that were secreted by the MDA-MB-231 cells; however, it did not affect cell viability over the 0.1 to 10.0 μM concentrations used in this study. The low cytotoxicity of batimastat and MMP inhibition by batimastat over this range of concentrations has

also been observed for human MDA-MB-435 breast cancer cells (28). The results presented here confirm the separate observations regarding invadopodial proteolysis and invasion of human breast cancer cells reported by others (8, 20) and because both parameters were observed and manipulated in the same study it now demonstrates the importance of invadopodial proteolysis of extracellular matrix to the invasion process.

MMPs play a critical role in mediating the invadopodial proteolysis of extracellular matrix that facilitates tumor cell invasion. This is evidenced by the effectiveness of the MMP inhibitor batimastat in simultaneously reducing invadopodial proteolytic activity and invasiveness of malignant breast cells. A number of MMPs have been implicated in having a role in proteolysis of extracellular matrix by human breast cancers including: MMP-2 (gelatinase A) (29, 30), MMP-9 (gelatinase B) (2, 31, 32), MMP-11 (stromelysin-3) (33), an 80 kDa MMP (34) and MMP-14 (MT1-MMP) (35). Several of these MMPs are known to be inhibited by batimastat including MMP-2, MMP-9, MMP-11 and possibly MMP-14 (36). In this study, the importance of MMPs in breast cancer cell invasion may be somewhat over-emphasized by the invasion assay used because the triple helical type I collagen comprising the gels is resistant to proteolytic cleavage by most proteases except MMPs (2). Thus, although there is mounting evidence that MMPs have a critical role in tumor cell invasion into the complex basement membrane and stromal matrices within living organisms, the evidence also suggests that

coordinated activity of other proteases and glycosidases in addition to MMPs is needed to efficiently degrade the matrix and facilitate enable invasion (1). For example, an *in vitro* study showed that in the presence of serum, batimastat only inhibited MDA-MB-231 proteolysis of human endothelial basement membranes by 30 % but an additional 30-40 % inhibition of basement membrane degradation was achieved when batimastat was used in combination with inhibitors of urokinase type plasminogen activators (37). However, even though there is an apparent need for other classes of proteases to efficiently degrade extracellular matrices *in vitro*, batimastat inhibition of MMPs has been effective in reducing the growth and spread of mammary tumors in animal models (28, 38). Thus development of MMP-inhibitors for use in anti-tumor therapies continues to be a promising area of research (36).

Elucidation of the mechanisms that promote concentration of MMPs and other extracellular matrix-degrading proteases to invadopodia may lead to new ways to identify malignant cells and inhibit their invasion. MMP-2 and other matrix-degrading proteases are not restricted to invadopodia. MMP-2 is secreted as a soluble enzyme that can be found within the cytoplasm of cells and embedded in the extracellular matrix as well as on invadopodia. The integral membrane MMP, MT-MMP-1 has been identified as an activator of latent MMP-2 and proposed to serve as a cell surface receptor for MMP-2 (39). In addition, the $\alpha_v\beta_3$ integrin has also been shown to serve as a cell surface receptor for MMP-2 (40). MT-MMPs or

integrins or both of these molecules may have a role in concentrating MMP-2 to invadopodial membranes of human breast cancer cells. It has been shown that MT1-MMP must be localized to invadopodia to stimulate the local degradation of extracellular matrix that is characteristic of invadopodia and that its cytoplasmic domain has a role in directing MT1-MMP to invadopodial membranes (12). Another mechanism for recruitment of active proteases to invadopodial membranes could involve directed oligomerization of the subunits of integral membrane proteases such as seprase, fibroblast activation protein-α, dipeptidyl peptidase IV and meprin (3). This directed oligomerization might occur in response to signalling through integrins (19). Perturbing the recruitment of proteases and their assembly onto the invadopodial surface might effectively reduce invadopodial proteolysis of extracellular matrix and inhibit invasion.

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Table I. Cell Viability

Cell line and treatment	% Viable
HBL-100	99.1
HBL-100 + DMSO	97.4
MDA-MB-231	99.6
MDA-MB-231 + DMSO	99.7
MDA-MB-231 + 0.1 μM BB-94	98.0
MDA-MB-231 + 1.0 μM BB-94	99.1
MDA-MB-231 + 10.0 μM BB-94	99.02

Table Legend

Table 1. Batimastat is not cytotoxic to the normal or malignant human breast cells. The values given are the percentage of viable cells in the cultures exposed for 72 h to growth medium that was 10 % (v/v) with respect to MMP/TIMP and plasminogen/plasmin-depleted fetal bovine serum (HBL-100, MDA-MB-231), the same medium with added DMSO (HBL-100 + DMSO, MDA-MB-231 + DMSO) or increasing concentrations of batimastat (MDA-MB-231 + 0.1 μM BB-94; MDA-MB-231 + 1.0 μM BB-94; MDA-MB-231 + 10.0 μM BB-94). Viabilities were determined using a trypan blue exclusion assay. Neither the DMSO solvent nor the batimastat were cytotoxic.

Figure Legends

Figure 1. Normal HBL-100 human breast cells do not degrade fibronectin but malignant MDA-MB-231 human breast cancer cells degrade fibronectin. Normal HBL-100 cells (A) and the fibronectin substrate (B) in the same microscopic fields are visualized by rhodamine phalloidin and fluorescein-fibronectin fluorescence respectively. Arrowheads point to identical locations in the microscopic fields revealing the cells (A) and the uniformly fluorescent fibronectin underneath the normal cells (B). Malignant MDA-MB-231 human breast cells (C) degrade fibronectin that is covalently linked to the substrate (D). Arrowheads point to identical locations in the same microscopic field revealing the cells (C) and fluorescence-negative spots where the fibronectin has been removed (Arrowheads: D). The observed focal degradation of fibronectin is characteristic of invadopodia mediated proteolysis. Bar = 10 μm.

Figure 2. Inhibition of MDA-MB-231 invadopodial matrix degradation by batimastat. Cells (A, C & E) and underlying substrates (B, D & F) in the same microscopic fields (A:B, C:D, & E:F) are visualized respectively for rhodamine phalloidin (cells) and fluorescein-fibronectin (substrates) after growing 24 h. Proteolytic degradation of fluorescent fibronectin substrates associated with invadopodia is detected in control cells (Arrows panel A & B) but not in cells treated

with 0.1 μM (C & D) or 1.0 μM batimastat (E & F). Batimastat treated cells have normal morphology (C & E). Bar = 10 μM.

Figure 3. Quantification of matrix proteolysis by normal and malignant human breast cells.

A) Malignant MDA-MB-231 human breast cells (■) release up to 11 fold more fibronectin into the media than the normal HBL-100 human breast cells (●) over 72 h. Levels of radioactivity were determined in two separate experiments. Each time point is plotted to the average of the two determinations after background subtraction.

B) Inhibition of MDA-MB-231 human breast cancer invadopodial proteolysis of matrix by the metalloproteinase inhibitor batimastat. MDA-MB-231 human breast cancer cells degrade ^{125}I-fibronectin in the absence (···□···) or presence of the DMSO (-■-) vehicle. Batimastat reduces proteolysis of the ^{125}I-fibronectin substrate by MDA-MB-231 human breast cancer cells to below that of control experiments without cells (-□-). Results are expressed as percentage of substrate released and plotted to the average of determinations made in two separate experiments. Each time point is plotted to the average of the two determinations after background subtraction. **Inset:** The left panel is a zymogram showing gelatinase activities at 92 kDa and 72 kDa in protease-depleted growth medium conditioned by MDA-MB-231 cells. The right panel is a zymogram of the same sample incubated with 10 μM

batimastat causing loss of the 92 kDa and 72 kDa gelatinase activities.

Figure 4. MDA-MB-231 human breast cancer cells invade into type I collagen gels.

A) Invasion of different human breast cell lines into type I collagen gels. The percentage of cells that invade into type I collagen after 72 h was determined for three different human breast cell lines. The results indicate that the HBL-100 normal human breast cell line (H100) is least invasive, the MCF-7 human breast cancer cell line (MCF7) is moderately invasive, and the MDA-MB-231 human breast cancer cell line (M231) is most invasive. For each cell line, the graph is drawn to the average value of 16 different determinations obtained in eight separate experiments. Bars indicate ± standard error.

B) Depth of the leading front of invasion into type I collagen gels of different human breast cell lines. The depth of the leading front of cells invading into type I collagen gels was determined after 72 h for three human breast cell lines. The results indicate that HBL-100 cells (H100) invade least deeply into the type I collagen gels, MCF-7 cells invade to a moderate depth (MCF7) and MDA-MB-231 cells invade furthest into the type I collagen gels (M231). For each cell line, the graph is drawn to the average value of 40 determinations obtained in the same eight experiments reported in C. Bars are drawn to ± the standard error.

C) MDA-MB-231 human breast cancer cells growing on top of a type I collagen gel. Cells (5×10^4) were seeded on top of the gel and allowed to invade

for 72 h. In this panel, the plane of focus is the top of the gel.

D) Three MDA-MB-231 cells (arrows) are shown invading 600 μm into the type I collagen gel. They were located by focusing down from the top of the gel in the microscopic field shown in panel A. Scale bar for panels A & B is shown in panel B = 100 μm.

Figure 5. Batimastat inhibits the invasive behavior of MDA-MB-231 human breast cancer cells.

The percentage of HBL-100 cells (H100) and MDA-MB-231 cells (M231) invading into type I collagen gels (panel A) and the leading front of invading cells (panel B) for two different experiments are shown (A & B: hatched bars = experiment 1, clear bars = experiment 2).

A) Batimastat inhibits invasion of MDA-MB-231 breast cancer cells into type I collagen gels. Batimastat (1.0 μM) inhibited invasion up to ten fold relative to DMSO control. Increased batimastat concentrations resulted in decreased invasion (compare in Experiment 1 (hatched bars): M231 + 0.1 μM Bat & M231 + 1.0 μM Bat and in Experiment 2 (clear bars): M231 + 1 μM Bat & M231 + 10 μM Bat). The graphs are the average of two data points and the bars indicate the range of the determinations.

B) Batimastat reduces the distance traveled into the type I collagen gel by cells at the leading front of invasion. In experiment 1 (hatched bars), the leading

front of invasion was reduced 2.2-fold by 1.0 μM batimastat (M231 + 1.0 μM Bat) and 2.9-fold by 0.1 μM batimastat. In experiment 2 (clear bars), invading cells were not detected (*) below the top of the type I collagen gel in the batimastat treated groups (M231 + 10 μM Bat* & M231 + 1.0 μM Bat*). The graphs are averages of measurements of the leading front of invasion at five locations within the gel and bars represent ± standard error.

Figure 1

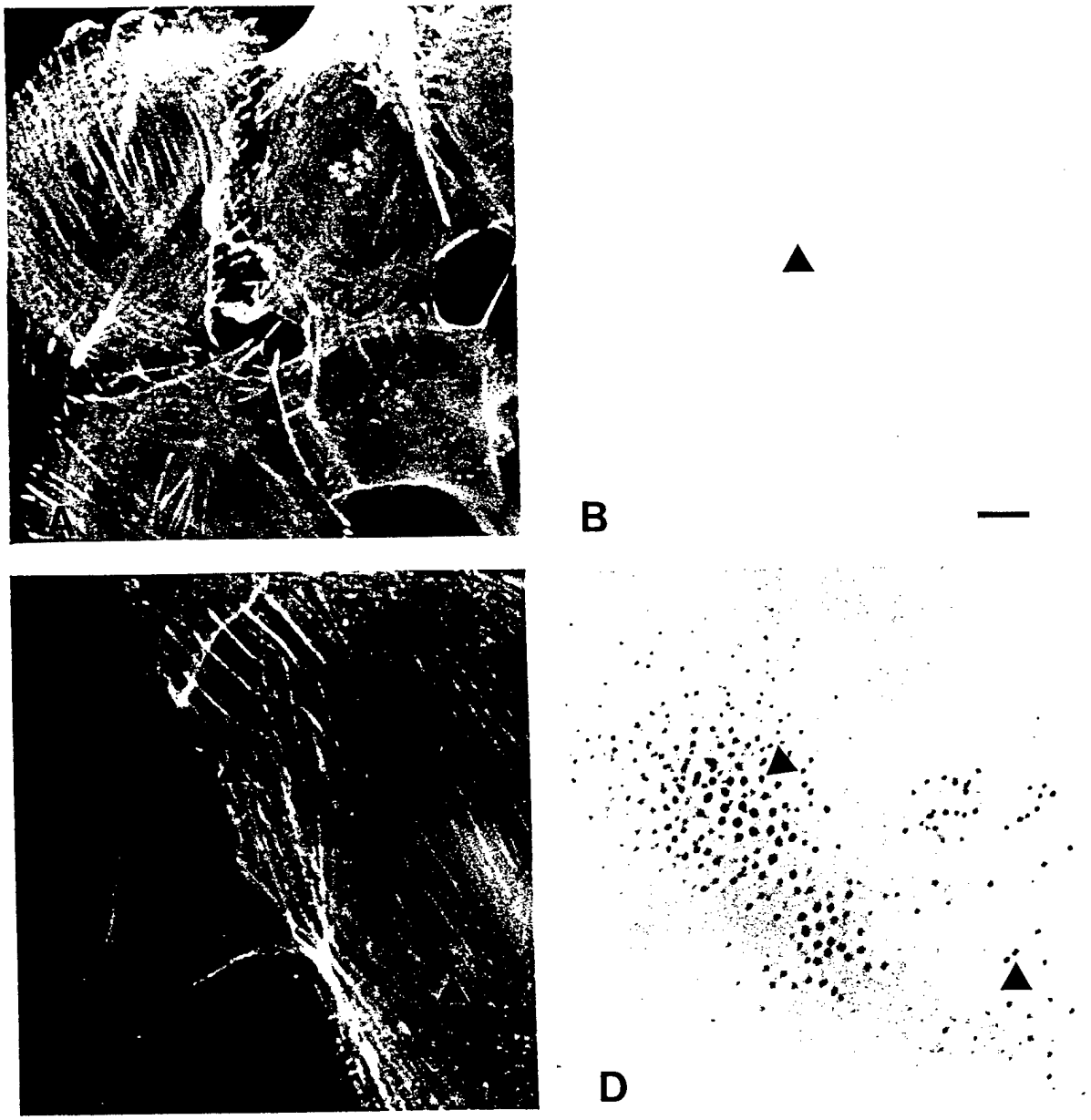


Figure 2

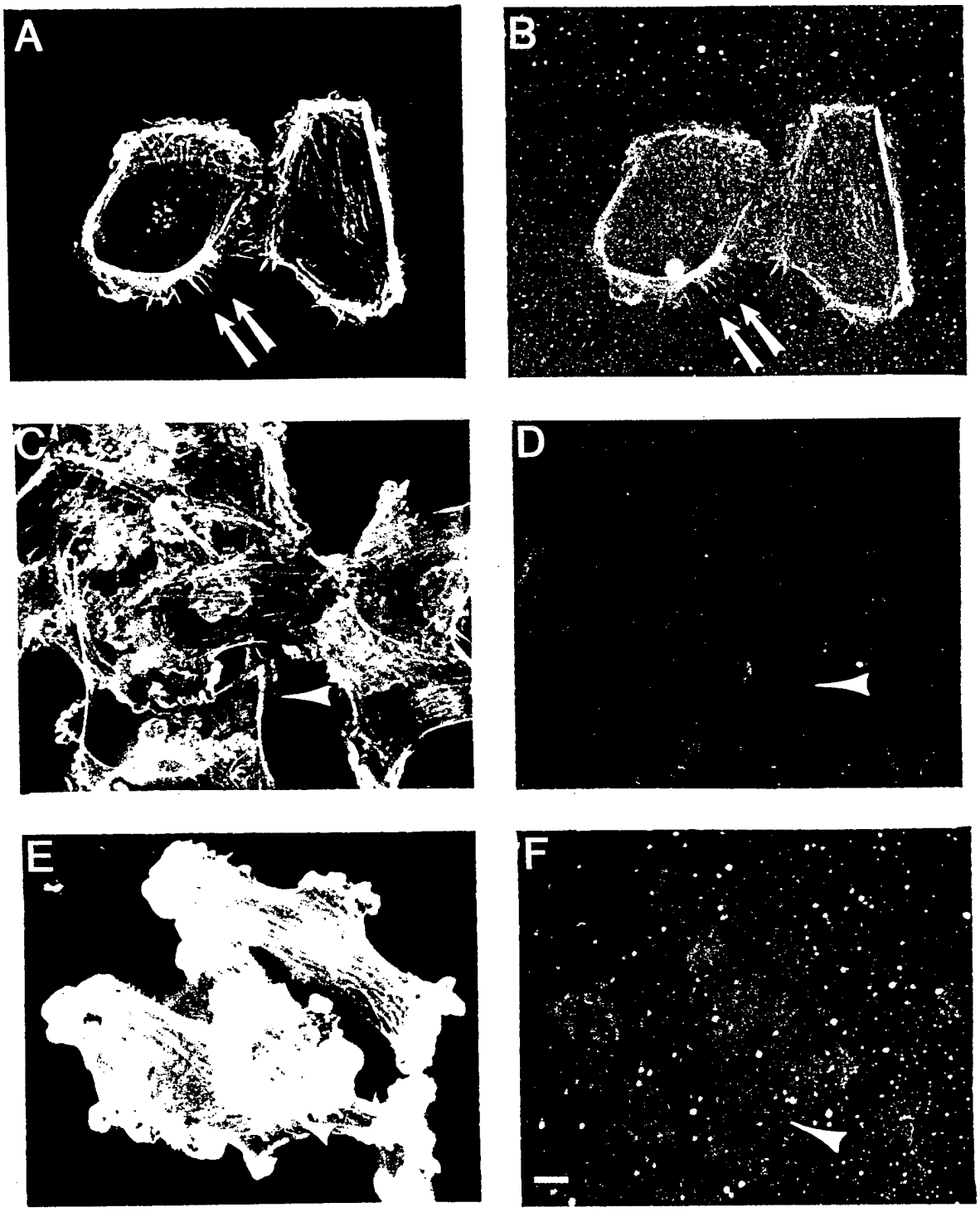


Figure 3

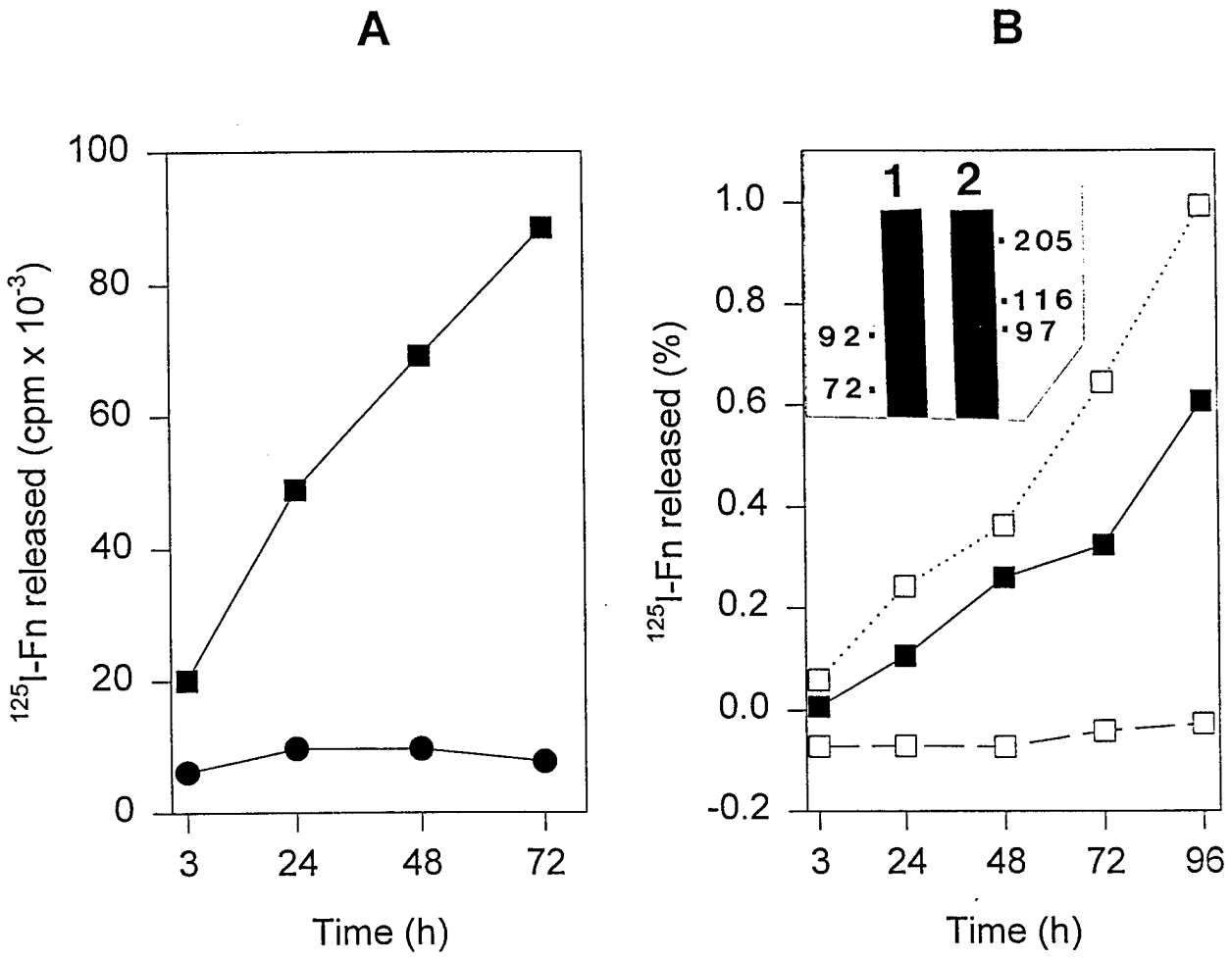
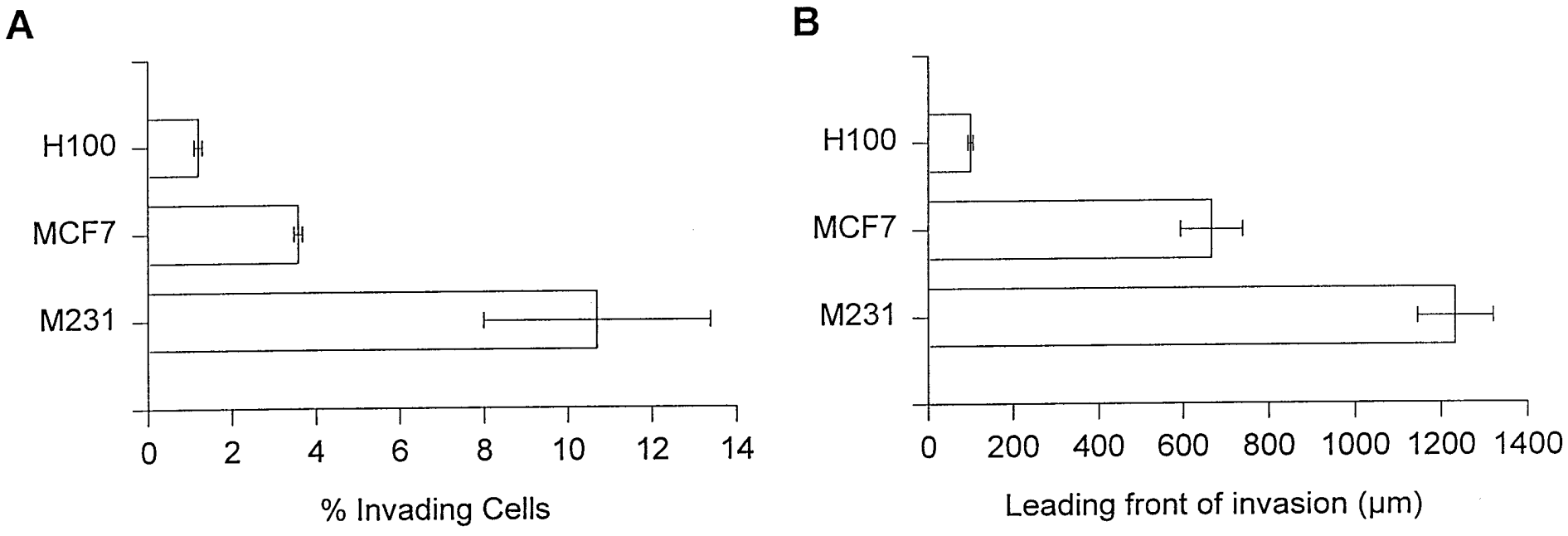
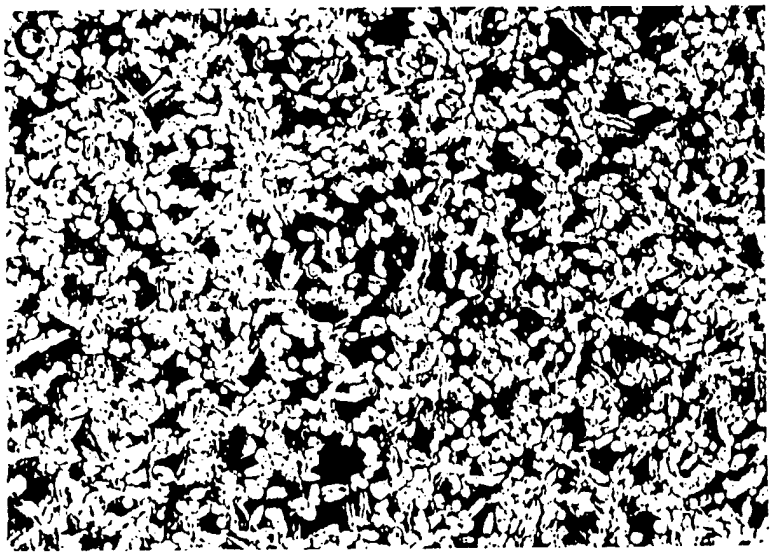
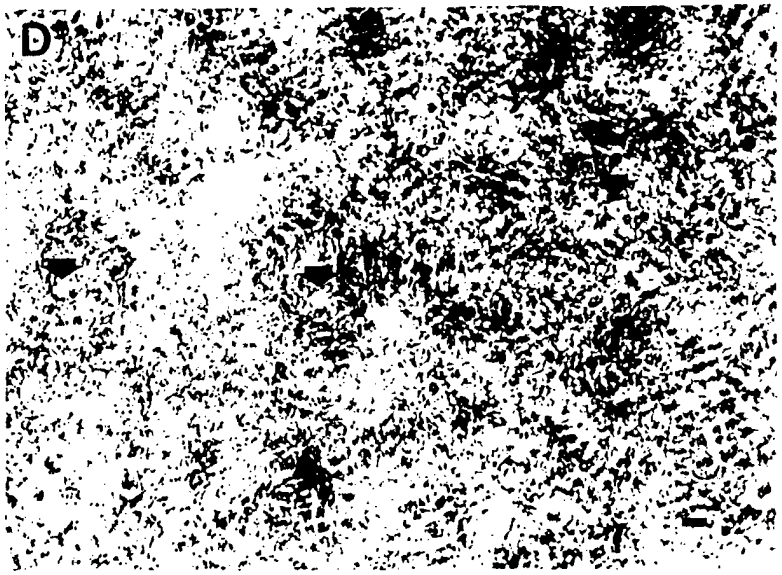

A
B
100
80
60
40
20
0
^{125}I-Fn released (cpm x 10^{-3})
3
24
48
72
Time (h)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
^{125}I-Fn released (%)
1
2
205
116
97
92
72
3
24
48
72
96
Time (h)

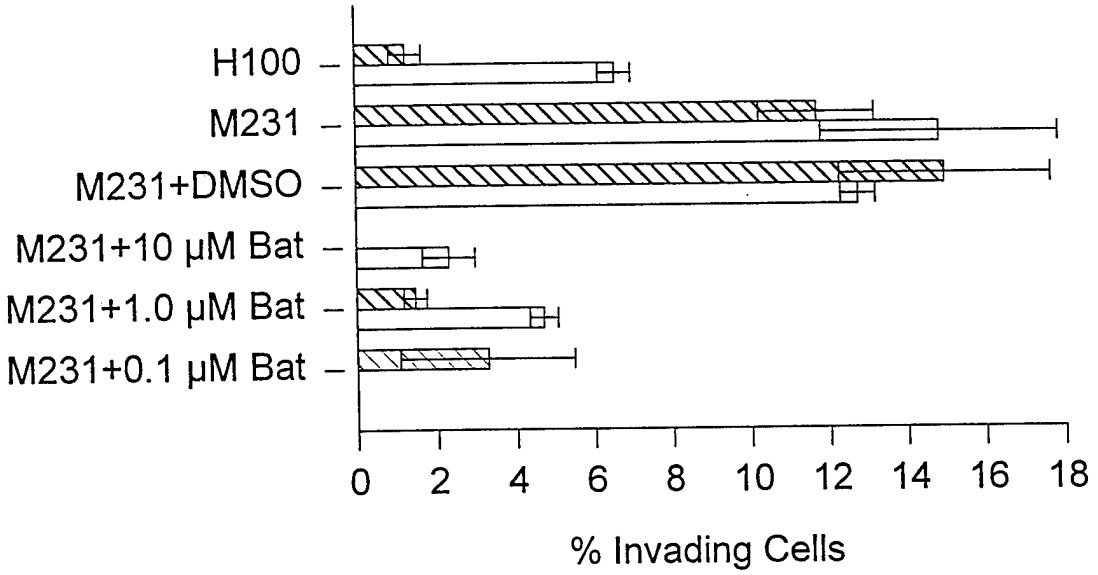
Figure 4


A
H100
MCF7
M231
0
2
4
6
8
10
12
14
% Invading Cells
B
H100
MCF7
M231
0
200
400
600
800
1000
1200
1400
Leading front of invasion (µm)


C


D

A


H100
M231
M231+DMSO
M231+10 μM Bat
M231+1.0 μM Bat
M231+0.1 μM Bat
0
2
4
6
8
10
12
14
16
18
% Invading Cells

B

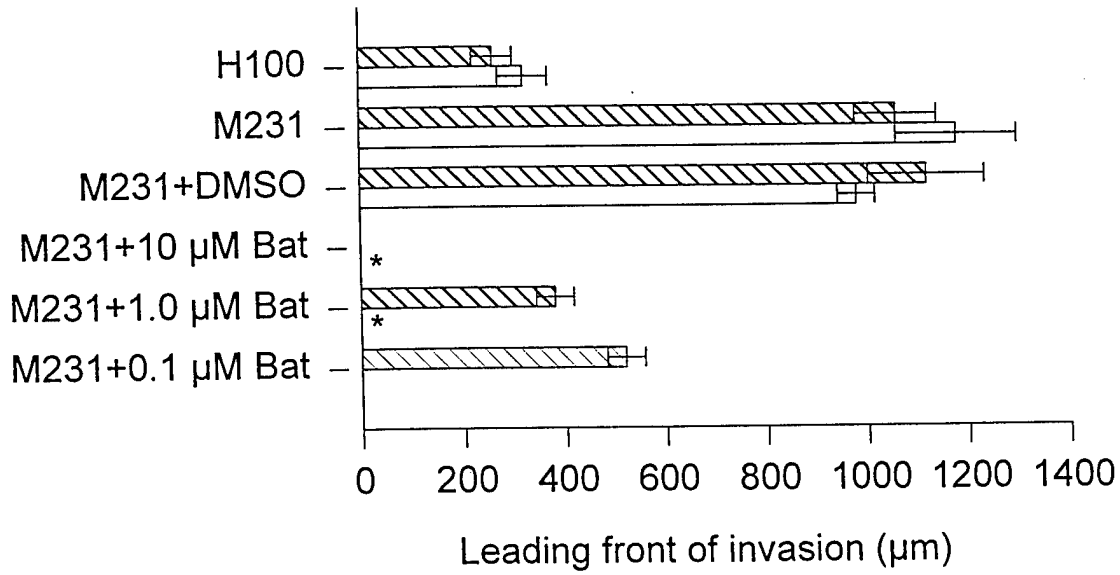

H100
M231
M231+DMSO
M231+10 μM Bat
M231+1.0 μM Bat
M231+0.1 μM Bat
*
*
0
200
400
600
800
1000
1200
1400
Leading front of invasion (μm)

Figure 5